The Biophysical Effects of an N-terminal Extension on Tumor Suppressor p53

Honors Research Thesis

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By

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Abstract

Tumor suppressor p53 is a transcription factor that is central to a system of cell cycle regulation and the prevention of cancer. The *C. elegans* p53 homolog CEP1 is more stable than human p53. It has been hypothesized that this is due in large part to a specific segment known as the S7S8 turn. The S7S8 turn is ten residues in human p53 but the analogous structure is only six amino acids in *C. elegans* p53. Because the turn is shorter and likely less flexible in *C. elegans* p53 than in human p53, it is hypothesized that this elongated turn in human p53 lends to increased movement and instability. Therefore, shortening the S7S8 turn in p53 might stabilize the turn and the entire protein. In addition to the S7S8 turn, a focus of this research is centered on understanding the effects of adding an N-terminal extension to the core domain of p53. This segment was initially added for the purpose of reducing aggregation. However, initial experiments suggest that this segment might also affect stability and function. Two sets of a panel of eight turn variants with differing sizes and physical properties were synthesized to compare the variants with the N-terminal extension and the ones without it. The Magliery lab has developed a screen which tests the DNA-binding ability of p53. The screen was made with green fluorescent protein that is negatively suppressed in the presence of active p53 protein. Because none of the mutations made to our p53 mutants are in the DNA-binding region, it is hypothesized that any variation in fluorescence is due to differences in stability. Western blots were performed to further probe the screen and test this hypothesis. Finally, circular dichroism, differential scanning fluorimetry, and thermal melts were used to distinguish biophysical differences between various turn mutants with and without the N-terminal extension.

Acknowledgments

I would like to thank my advisor, Dr. Tom Magliery, for giving me the privilege to work in his lab as an undergraduate. I want to thank David Bowles for training me, providing guidance, and for being a good friend. I also want to thank Sid Mohan and Kiran Doddapaneni for their assistance in running Western blots. I would like to thank Dr. Marcos Sotomayor and Dr. Quintin Pan for serving on my defense committee. I would be remiss to go without thanking all of the undergraduates for making my lab experience enjoyable and, indeed, the entire lab, for assisting all of us.

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Major Field: Biochemistry

Minor Field: French

Minor Field: Society & Health

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Introduction

A brief history of p53 and cancer

The history of cancer research is long. In the 1860s, Virchow identified the connection between cancer cells and increased inflammation[1]. In the 1950s, Otto Warburg theorized that it was a change from cell respiration to fermentation that caused cancer cells[2]. Monumental discoveries of the importance and structure of DNA led to the modern consensus that tumorigenic cells are initiated by DNA mutation. In 2012, GLOBOCAN estimated that there about 14.1 million new cancer patients and 8.2 million deaths worldwide[3].

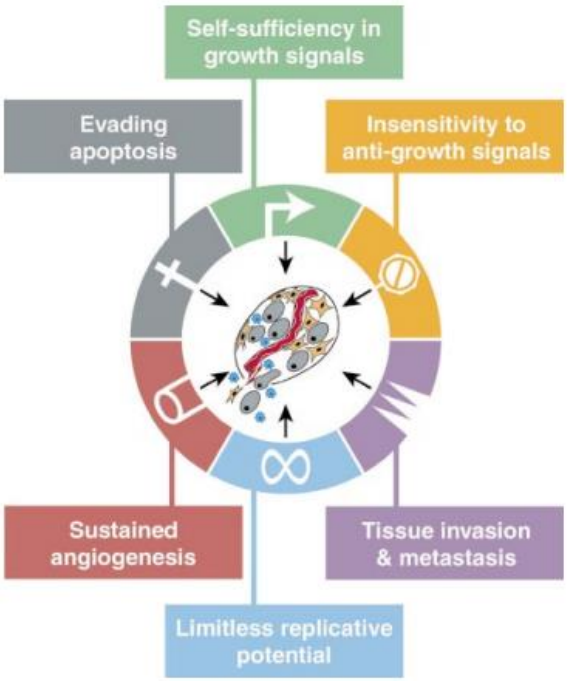

Figure 1: The six hallmark capabilities of cancer[4]. Tumor suppressor p53, when functional, can prevent a cancerous cell from replicating or can cause apoptosis.

p53 was first identified in 1979 and then classified as an oncogene or a cancer-causing agent[5]. It was later theorized that p53 was a tumor suppressor. The fact that p53 protein was found to be

functional in healthy cells while dysfunctional in cancer cells, through protein unfolding or an inability to bind to DNA, supported the second theory. It has recently been shown that the truth lies somewhere between these two theories. Healthy p53 protein does appear to function as a transcription factor and tumor suppressor[6]. However, some mutant p53 proteins have been shown to be carcinogenic themselves[7, 8, 9].

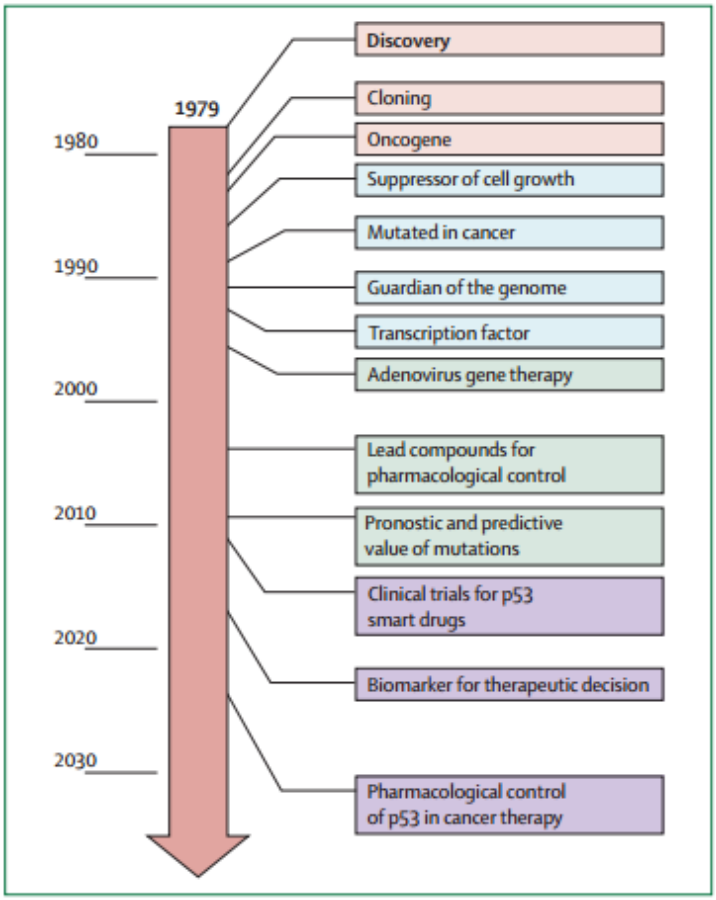

Figure 2: p53 research timeline[10]. Pink: early steps in understanding p53 function (1979-1988). Blue: p53 as a tumor suppressor and transcription factor, protecting against oncogenic DNA damage (1988-1994). Green: current research focusing on p53 mutations in cancer and development of drugs targeting p53 (1995-present). Purple: projected applications of p53 research to cancer diagnosis and treatment.

Loss of function of tumor suppressor p53 opens the door for carcinogens to alter a cell's DNA towards a cancerous state. p53 mutations can also lead to gain of function of oncogenic properties, but this is not necessarily true (in fact, it may be rare).

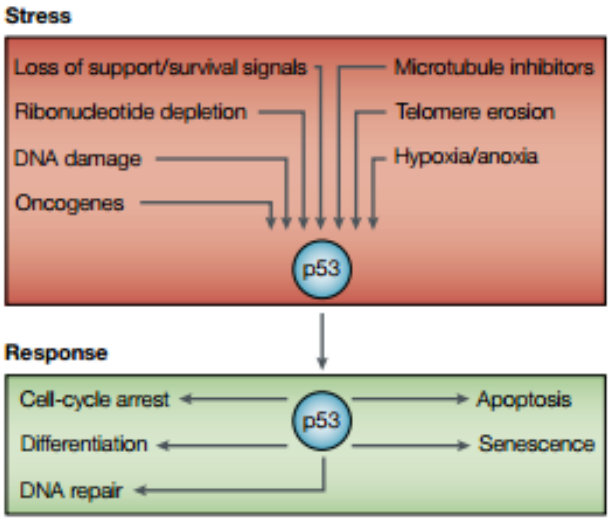

Figure 3[11]: A list of stressors that cause p53 to respond. These signals usually lead to stabilization of p53 protein, which leads to an increase in cellular p53 levels. The effect of p53 activation is generally to inhibit cell growth, but cell-cycle arrest or induction of apoptosis can also occur in order to prevent tumor development.

Upon reading any scientific paper on tumor suppressor p53, one would have to struggle to find a paper that does not include the fact that "mutated p53 is found in more than half of all human cancer" and the phrase "guardian of the human genome"[10, 12]. Tumor Suppressor p53 is referred to as the guardian of the human genome because it is viewed as central to the cell's defense mechanisms to prevent cancer development. In my opinion, p53 has been recognized as the guardian of the human genome thanks to a paradox. Rather than being a strong link and dependable protein (as the word guardian would suggest), p53 tends to fail so that it is often the

cause of the breakdown of the cell's defense mechanism. In fact, the p53 gene is the most frequently mutated gene in human cancer[13]. This is because p53 has evolved to be an inherently unstable protein[14-15]. Singular mutations, such as V143A, R175H, and R273H destroy its functionality[16]. This is why it is our goal to discover a method to raise the stability of the p53 protein. How this is done is the focus of this research.

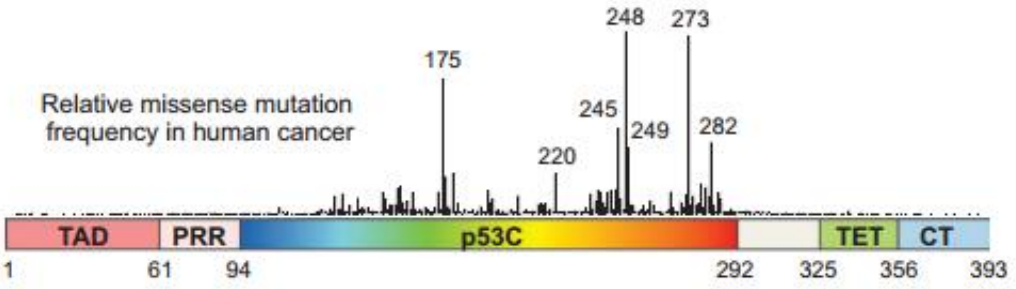

Figure 4[17]: The most common p53 missense mutation in human tumors. About 95% percent of the mutations lie within the core DNA-binding domain[18] (Brown et al – 2009). 75% of these mutations are missense mutations.

Tumor Suppressor p53 works among a system of factors, such as MDM2 and MDM4, which influence gene transcription[19]. When activated, p53 prevents cancer development via DNA repair, senescence, or apoptosis[20].

S7S8 Turn

The Nussinov lab conducted molecular dynamics simulations, comparing human p53 protein to its homolog in *Caenorhabditis elegans*. The simulations predicted that the worm's p53 homolog (Cep-1) was more stable than human because of three major differences: Loop L1, the turn between helix H1 and beta-strand S5, and the S7S8 Turn. Loop L1 lacks secondary structure and

both turns are much longer in the human p53[21]. The Nussnov lab designed a mutant by shorteninh the S7S8 turn and predicted that shortening the S7S8 turn should create not only a more stable S7S8 turn, but a more stable p53 protein overall. The goal of my research is to explore this hypothesis by creating several S7S8 turn mutants and compare their stability to the wild type p53 protein.

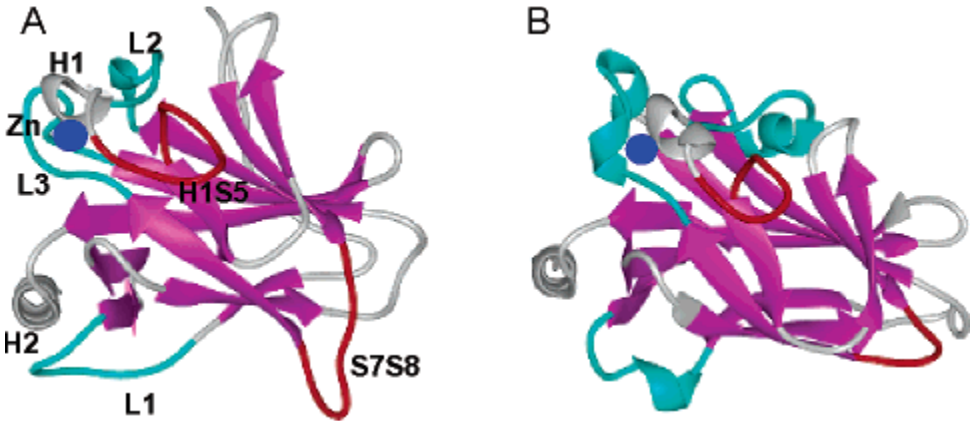

Figure 5[21]: Ribbon representations of crystal structures

2A: human p53 core domain; 2B: *Caenorhabditis elegans* homolog – Cep1.

This research focuses on the differences between the S7S8 turn in human p53 and its shorter and less flexible analogous structure in Cep1.

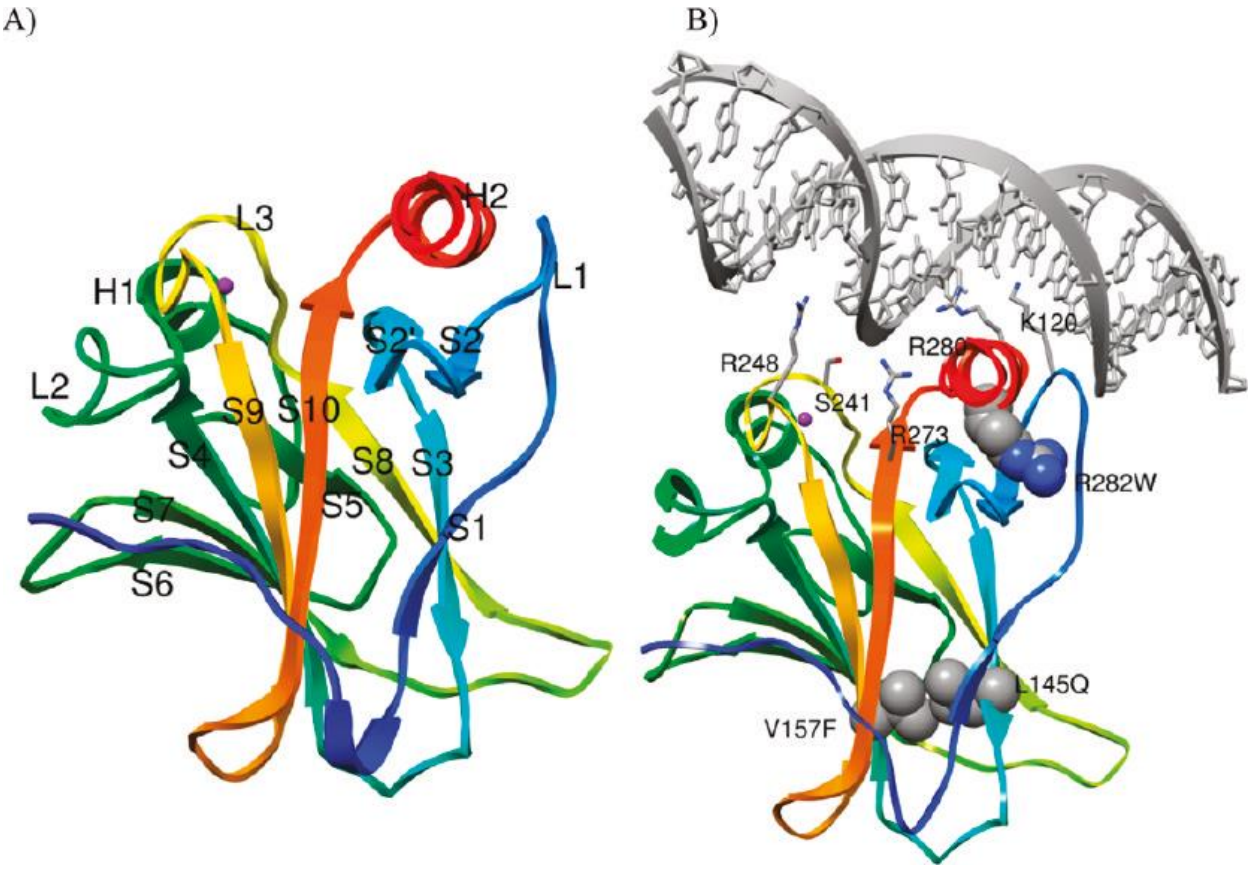

Figure 6[22]: p53 core domain structure.

6A: Ribbon diagram of DNA-free p53 core domain

6B: Ribbon diagram of p53 core domain bound to consensus DNA. Residues that contact DNA are shown as sticks, and mutation sites shown in space-filling representation.

Figure 5B is a good representation of how the p53 protein interacts with DNA as a transcription factor. Note that the S7S8 turn (at the bottom right of the diagram, colored green) is located away from the DNA. This is important when it comes to both stabilizing p53 and the screen created by the Magliery lab[23]. According to the Nussinov lab's simulations, the three regions that one could target to stabilize p53 would be the L1 loop, S7S8 turn, and H1S5 turn[21]. The Fersht lab demonstrated that stabilizing the L1 loop can stabilize p53[24]. The S7S8 turn, however, is further away from the DNA binding region of the protein so the turn can be altered without

impacting DNA binding as much as altering the L1 loop would. This is a strong reason for focusing on stabilization of the S7S8 turn to increase the overall stability of p53 rather than the L1 loop or H1S5 turn. In regards to the Magliery lab screen, it tests for DNA binding, not stability. However, because the changes to our S7S8 turn variants should not impact DNA binding, the only differences seen should reflect changes in stability (note: it could be affected by aggregation, which would be clearer with a Western Blot).

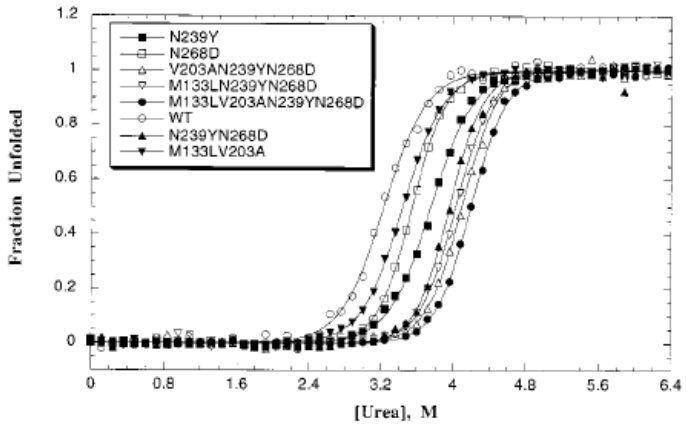

Figure 7[25]: This urea melt originated in an instrumental paper in the Fersht lab. It demonstrated that the p53 core domain could be stabilized by mutations in general and four specific ones – M133L, V203A, N239Y, N268D – in particular. The p53 protein with these four mutations is known as the Quadruple mutant, or Quad for short.

N-Terminal Extension

In 2011 the Fersht lab made a finding concerning an N-terminal extension to the core domain of human p53. p53 protein aggregates rapidly which can render protein analysis difficult, which is why it was a welcome sign to find that adding an extension of five residues (sequence: PSWPL)

onto the N-terminus of the DNA-binding domain of p53 greatly reduces aggregation[26]. The N-terminal extension reduces aggregation by capping a hydrophobic patch that was previously exposed to solvent. This is mainly due to a cation-Pi interaction of indole ring of Trp91 with the guanidinium group of Arg174. With this knowledge, we double are library of mutants to include both variants with and without the N-terminal extension. The goal is to measure if the N-terminal extension improves stability of the core domain in addition to reducing aggregation.

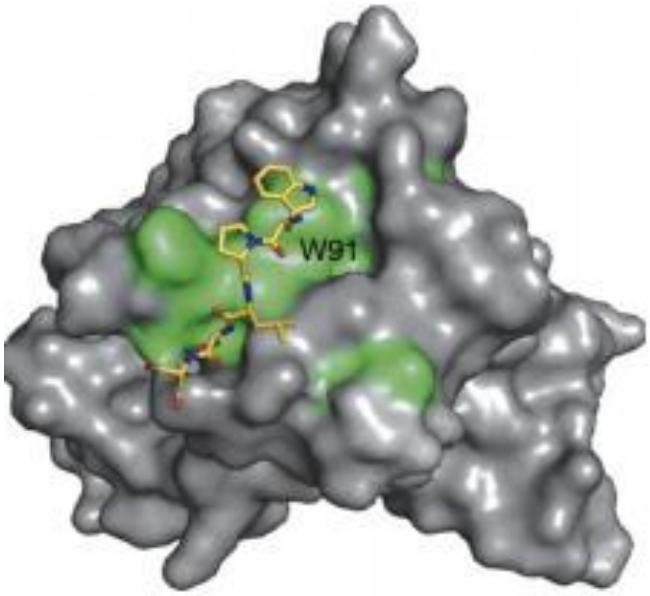

Figure 8[26]: Simulation of the N-terminal extension to the DNA-binding domain of p53 and how it acts as a caps a hydrophobic patch.

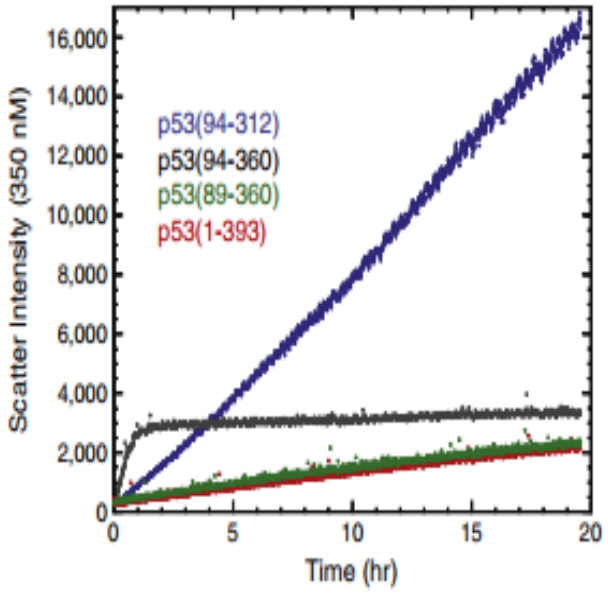

Figure 9[26]: Light scattering for various forms of p53. Increased light scattering indicates aggregation, so the marked decrease in aggregation from p53 (94-312) when adding the N-terminal extension for p53 (89-360) was a large impetus to adding the N-terminal extension to our variant.

In addition to reducing aggregation, the Fersht lab found that addition of the N-terminal extension raised the melting temperature of the core domain (89-293 vs 94-312) by 0.6 ^{o}C and raised the tetrameric p53 variants (residues 89-360 vs 94-360) by 2 ^{o}C. From these data, they concluded that the p53 DNA-binding domain is thermodynamically stabilized by the N-terminal extension. Our goal is to evaluate this claim that the N-terminal extension improves stability of the core domain.

Materials and Methods

Creation of Mutants

Name	S7S8 Turn Sequence
Human	YEPPEVGSDC
Cep1	MYPGA----V
AGSG	YA----GSGC
EASA	YE----ASAC
ESE	YE-----SEC
ESG	YE-----SGC
EGE	YE-----GEC
EGSG	YE----GSGC
EGSD	YE----GSDC
ESD	YE-----SDC

Table 1: Table of p53 S7S8 turn mutants and their sequences

*Note: This is not really the S7S8 turn in Cep1; however, it is the homologous structure.

Previous efforts in the Magliery lab were made to shorten the S7S8 turn in order to stabilize p53. These efforts include a complete removal of the turn and a transplant of the Cep1 sequence into p53 but neither was successful[27]. It was decided to shorten the turn to mimic the length of the analogous structure to the S7S8 turn in Cep1 and maintain the charged amino acids (Glutamate and Aspartate) due to the turn's solvent exposure and Glycine for geometrical reasons.

In addition to the S7S8 turn mutants in the table above, a series of negative mutants were created for screening and expression. They include V143A, R175H, E224Q, R249S, and R273H.

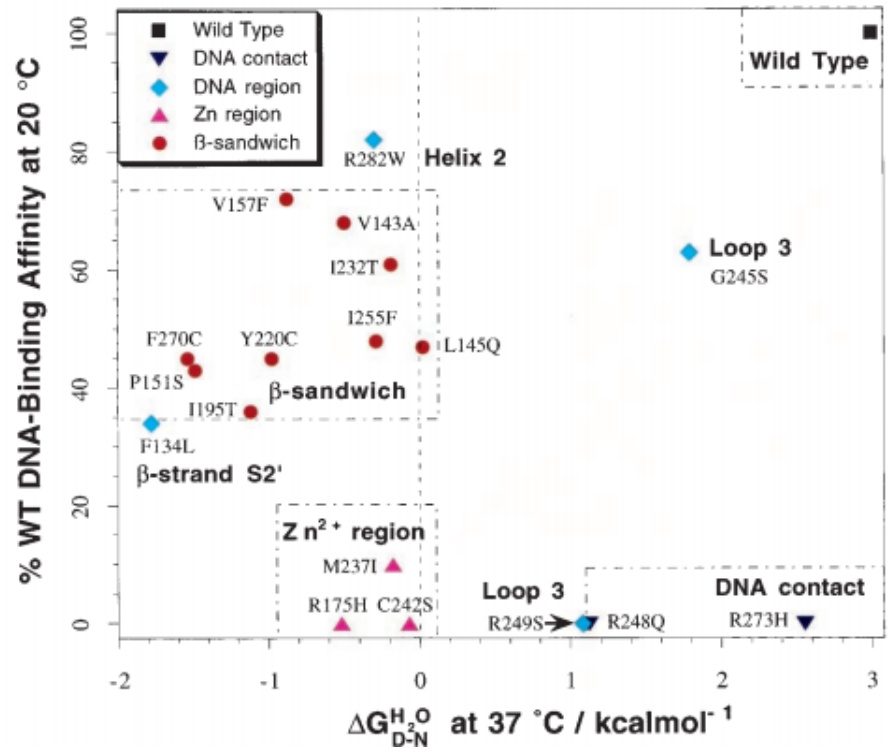

Figure 10: Plots the different effects the mutations have on p53's stability and binding affinity[16]. The mutants we cloned were chosen for their various effects, mostly negative. It is important to point out that if we are successful in stabilizing p53 by shortening and stabilizing the S7S8 turn, it would be a good target for mutants which have moderate DNA-binding affinity but reduced stability (e.g. V143A). However, it would not rescue mutants affecting DNA binding (e.g. R273H) or the Zn^{2+} region (e.g. R175H).

Cloning of Mutants

Mutants were cloned using Polymerase Chain Reaction. Conditions were optimized with recombinant Pfu polymerase. Mutagenic primers were ordered from Sigma Genosis and pre-

existing templates of p53 were used for mutagenesis. Potential contaminants were removed from PCR products via chloroform and ethanol extraction. To clone into the screening vector, pACBAD, PCR products were digested with restriction enzymes BamHI and BsrGI, and to clone into the expression vector, pHLIC, PCR products were digested with NcoI and XhoI. These digested inserts were purified with QIAQuick gel extraction and were then ligated at 16 ^{o}C overnight with the digested pACBAD vector for the screening strains and with digested pHLIC vector for the expression strains. The ligations (1 µL) were transformed into electrocompetent DH10β cells (30 µL), recovered for an hour and plated to the appropriate antibiotic plate. Single colonies were selected and grown to saturation, and the DNA was extracted, analyzed by restriction enzyme digest, and confirmed by DNA sequencing.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 ^{o}C	2 min	1
Denaturation	95 ^{o}C	30 s	25
Annealing	55 ^{o}C	30 s	
Elongation	72 ^{o}C	1 min	
Final Extension	72 ^{o}C	5 min	1
Soak	4 ^{o}C	N/A	1

Table 2: PCR Scheme

Screening

Brinda Ramasubramanian, a previous member of the Magliery lab, created an *in vivo* screen for functionality. The pACBAD p53 variants were co-transformed into DH10β electrocompetent cells with Green Fluorescent Protein and plated on LB Kan Amp plates for selection of single colonies. Colonies were selected, grown in 2YT to saturation, and plated on LB Kan Amp plates with 0.005% arabinose to induce p53 production. Fluorescence was measured with UV light after 48 hours of incubation at 37 ºC. Phenotypes were classified as passing the screen if the colonies were dark, failing the screen if the colonies were bright, and intermediate if their brightness was mediated.

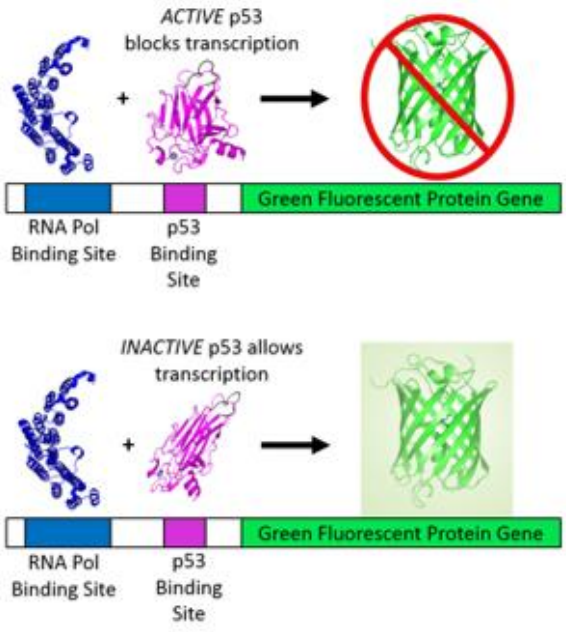

Figure 11: Schematic of cell-based screen created by Brinda Ramasubramanian. Cells will only pass the screen when p53 is active and bound to DNA, blocking the RNA polymerase's trajectory. If the p53 is stable but it does not bind, the colony will fluoresce. Or if the p53 is not stable, it will not bind to DNA and the colony will fluoresce.

Expression and Purification

After the variants were cloned into pHLIC and transformed into C41(DE3) cells, 25 mL seed cultures were grown overnight with Ampicillin. The seed was used to inoculate 1L of 2YT media (16 g tryptone, 10 g yeast extract, 5 g NaCl, 1 L H_2O), grown to OD600 0.6-1.0 and then induced with 0.2 mM IPGT (200 µL). The cultures were then grown for 16 hours at 25 ^oC then centrifuged at 5000 rpm for 10 minutes to create cell pellets and were frozen at -20 ^oC. The pellets were thawed and dissolved in 25 mL of lysis buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 10 mM imidazole, 100 µM $ZnCl_2$, 15 mM β-mercaptoethanol) at 4 ^oC. Once the mixture is homogeneous, the following were added on ice and incubated for 45 minutes: 1 mM PMSF (Phenyl-methyl-sulfonyl-flouride – 150 µL of 200 mM), 5 mM MgCl2 (75 µL of 2 M MgCl2), 0.5 mM CaCl2 (15 µL of 1 M Ca Cl2), DNase I (5 µL of 5 mg/mL), RNase A (5 µL of 10 mg/mL), and 10% Triton X-100 (300 µL). PMSF is needed as a protease inhibitor so the p53 protein does not degrade and the salts work as coenzymes for DNase I. Triton X-100 is a detergent used to solubilize proteins. The cells were lysed open by passing them through an Emulsiflex twice. The solution was then centrifuged at 15000 rpm for 1 hour (2x 1/2 hour spins) to retrieve the soluble p53 protein in the supernatant while the pellets were discarded. The supernatant was bound to Ni^{2+}-NTA resin (1 mL was buffer swapped into phosphate buffer) for 1 hour at 4 ^oC and then run through a large, pre-fitted column (BioRad). The column was washed with 6 mL lysis buffer containing 15 mM imidazole into 6 mL of p53 storage buffer (ingredients), then was washed with wash buffer, which was lysis buffer with 30 mM imidazole, and then with elution buffer, which was lysis buffer with 250 mM imidazole. The p53 protein was mostly in the first wash with lysis buffer. TEV protease was added at 16 ^oC repeatedly until SDS-PAGE gel indicated that the 6x His-tag had been cleaved. The solution was then re-bound

to Ni^{2+}-NTA resin (1 mL buffer swapped into phosphate buffer) and run through a column where the p53 protein would be in the flow through.

Biophysical Characterization

Differential Scanning Fluorimetry

19 µL of p53 protein samples were mixed with 1 µL of the diluted SYPRO Orange dye (Invitrogen) (provided as 5000x, diluted to 300x before addition, and then to 15x in the mixture) in 96-well thin-wall PCR plates (USA Scientific) and sealed with Adhesive PCR Film (Thermo Scientific). Using a BioRad C1000 Thermal Cycler, the temperature, starting at 12.5 ^{o}C, was increased by 0.2 ^{o}C every 12 seconds to acquire thermal denaturation curves.

Circular Dichroism

Data were collected on a Jasco J-815 CD spectrometer. Purified p53 protein samples were concentrated to 50 µM and any DTT was removed and replaced with TCEP by dialysis. For thermal denaturation, ellipticity was monitored at 222 nm with temperatures rising from 20 to 95 ^{o}C. Six samples were run for each p53 variant and the average melting temperature was recorded.

Western Blot

A 12.5% SDS-PAGE gel was run with rainbow ladder. The gel was then placed into a sandwich within a clamp in the following order: sponge – paper – gel – PVDF membrane – paper – sponge. The sandwich was made under Towbin buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, 10% Methanol) to and squeezed tightly to prevent air bubbles from forming. The sandwich

was transferred to a Western Blot transfer container and was filled with Towbin buffer and a 200 V current was applied for 80 minutes, making sure that the current was in the direction of leading the protein from the gel towards the PVDF membrane. An ice pack was placed in the container to prevent overheating and was replaced with a fresh ice pack if necessary. The membrane was removed from the sandwich and was washed with TBST (Tris Buffered Saline Tween – 25 mM Tris, 0.15 M NaCl, 0.05% Tween) for 10 min. The membrane was blocked for 1-2 hours with 30 mL 1% BSA (30 mL TBST + 0.3 g BSA) at room temperature. The membrane was then washed with TBST 2X 10 minutes.

The PVDF membrane was bound to the primary antibody (1:175 dilution – 30 mL TBST + 170 µg Pab240) overnight at 4 ^{o}C. Pab240 is a mouse monoclonal IgG_1 that targets residues 212-217 on the S7 strand[28] (Santa Cruz Biotechnology – catalog #sc-99; Friedler et al – Structural Distortion of p53).

The following morning the primary antibody was collected, as it can be used up to ten times, and the membrane was washed 2X 10 minutes with TBST at room temperature. Then the membrane was bound to the secondary antibody (1:500 dilution – 30 mL TBST + 60 µg secondary antibody) and incubated for 1 hour before washing again with TBST 2X 10 minutes. The secondary antibody was a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology – catalog #sc-2005). The Western was visualized via chemiluminesence and DAB.

Results

Cloning

Cloning of p53 was done with largely the same protocol as used by Ely Porter (Porter 2009)

A hurdle in my first year was finding the correct PCR conditions for cloning into pACBAD. The two agarose gels demonstrate how the denaturation and annealing temperatures were found.

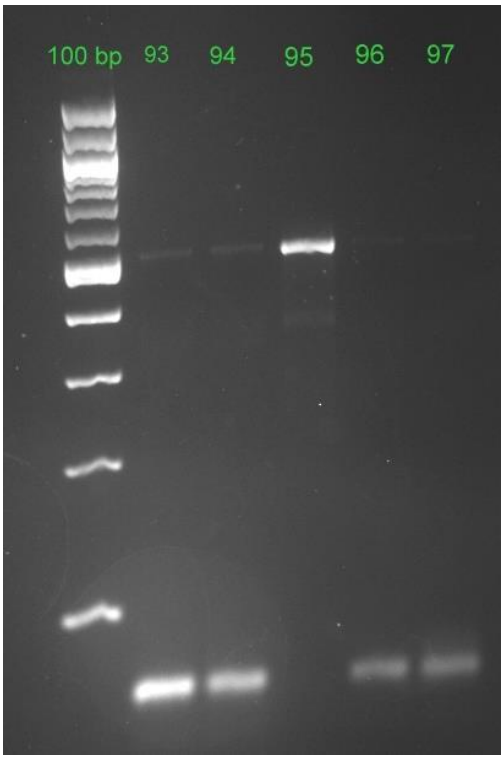

Figure 12: Agarose gel of PCRs with differing denaturation temperatures.

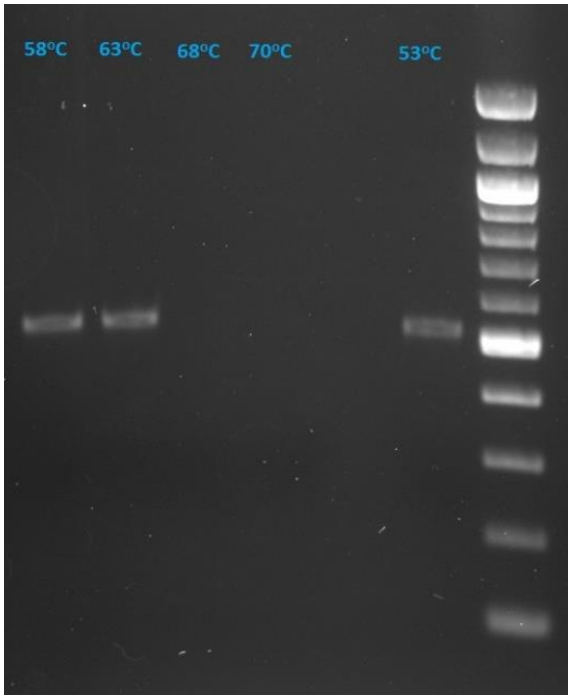

Figure 13: Agarose gel of PCRs with differing annealing temperatures.

PCR of p53 inserts

Figure 11 shows that 95 °C was the optimal denaturation temperature for PCR with pfu polymerase. Figure 12 illustrates that 63 °C and below was preferable as annealing temperatures for PCR with pfu polymerase. Temperatures that were too low were avoided in effort to prevent creation of unwanted side products. The general scheme used 50 °C as the annealing temperature, but if the PCR was unsuccessful, a gradient spanning 40-60 °C was introduced.

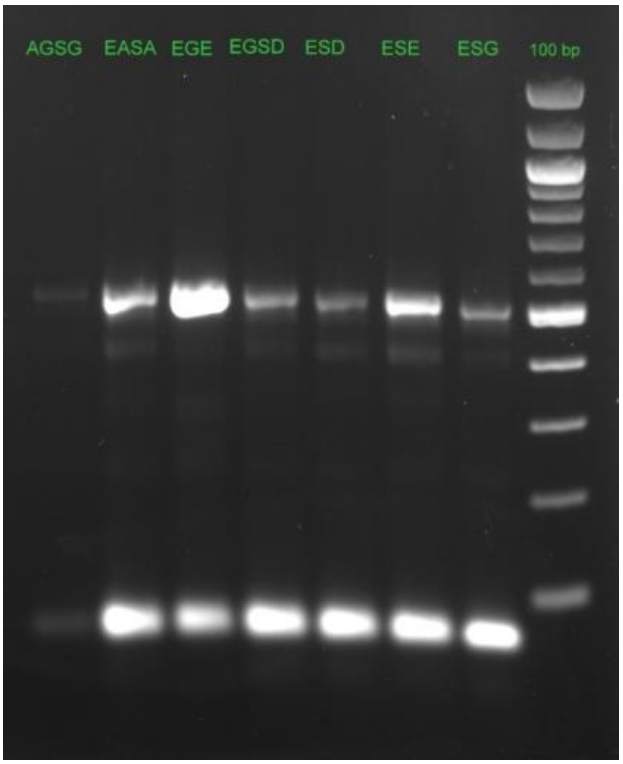

Figure 14: This agarose gel contains S7S8 turn mutant inserts after PCR and chloroform extraction. The insert is seen around the 500 bp band and the byproducts below were removed in QIAQuick gel extraction.

Enzyme Digest

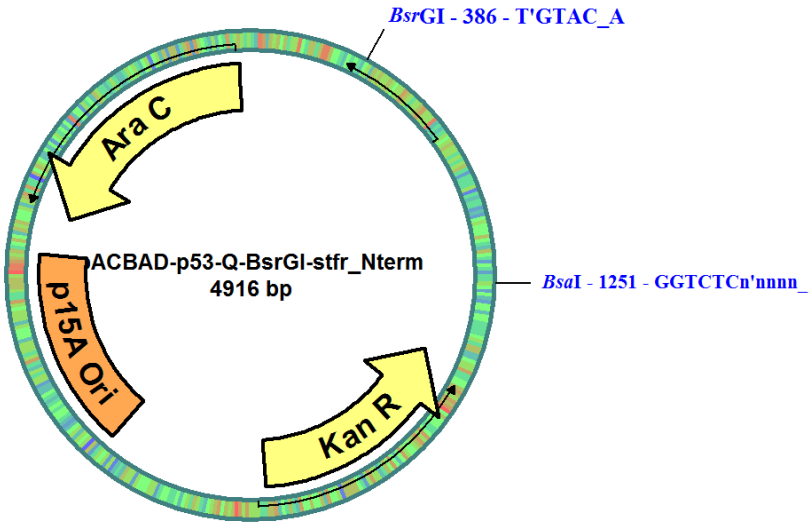

Figure 15: Plasmid map of pACBAD N-terminal Quad stuffer with BsaI and BsrGI cut sites. One key difference between cloning into pACBAD vs pHLIC is that the N-terminal extension is located within the vector for pACBAD while the extension is located within the insert for pHLIC.

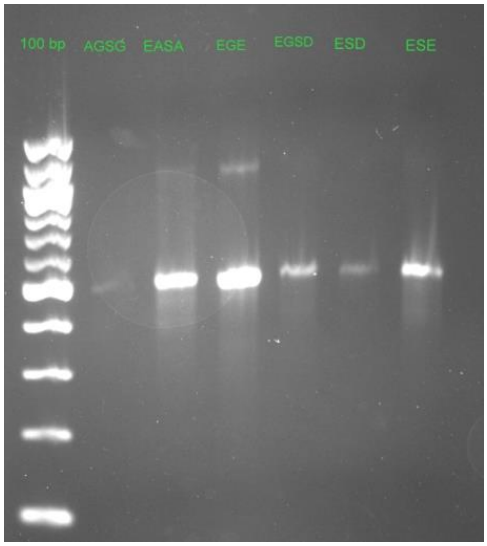

Figure 16: The agarose gel has the S7S8 turn mutant inserts after they were digested with BsaI and BsrGI for four hours and were purified by QIAQuick gel extraction.

Analytical Digest

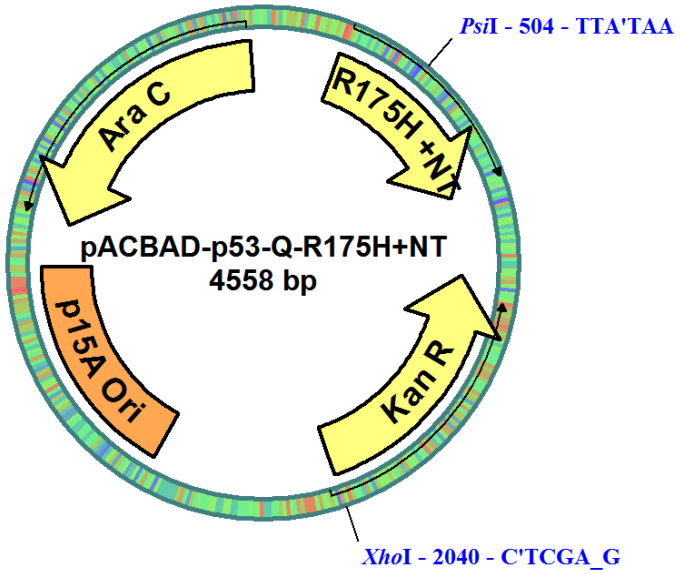

Figure 17: Plasmid map of p53 in pACBAD analytically digested with PsiI and XhoI.

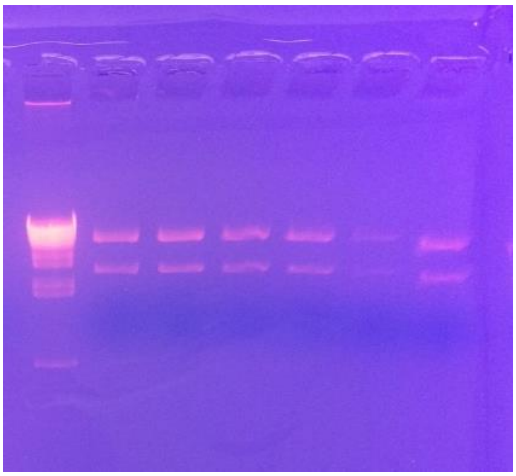

Figure 18: The agarose gel shows N-terminal EASA, EGE, EGSD, ESD, ESE, and ESG pACBAD mutants digested with PsiI and XhoI, resulting in two cuts. If the ligations had been unsuccessful, there would only be one band because there would be no PsiI site. After successful analytical digests such as this, variants were confirmed with DNA sequencing.

Cloning into pHLIC

Cloning variants into pHLIC was a very similar process to cloning into pACBAD. The only differences were to clone using diferent primers and digest with BamHI and NcoI.

Screen

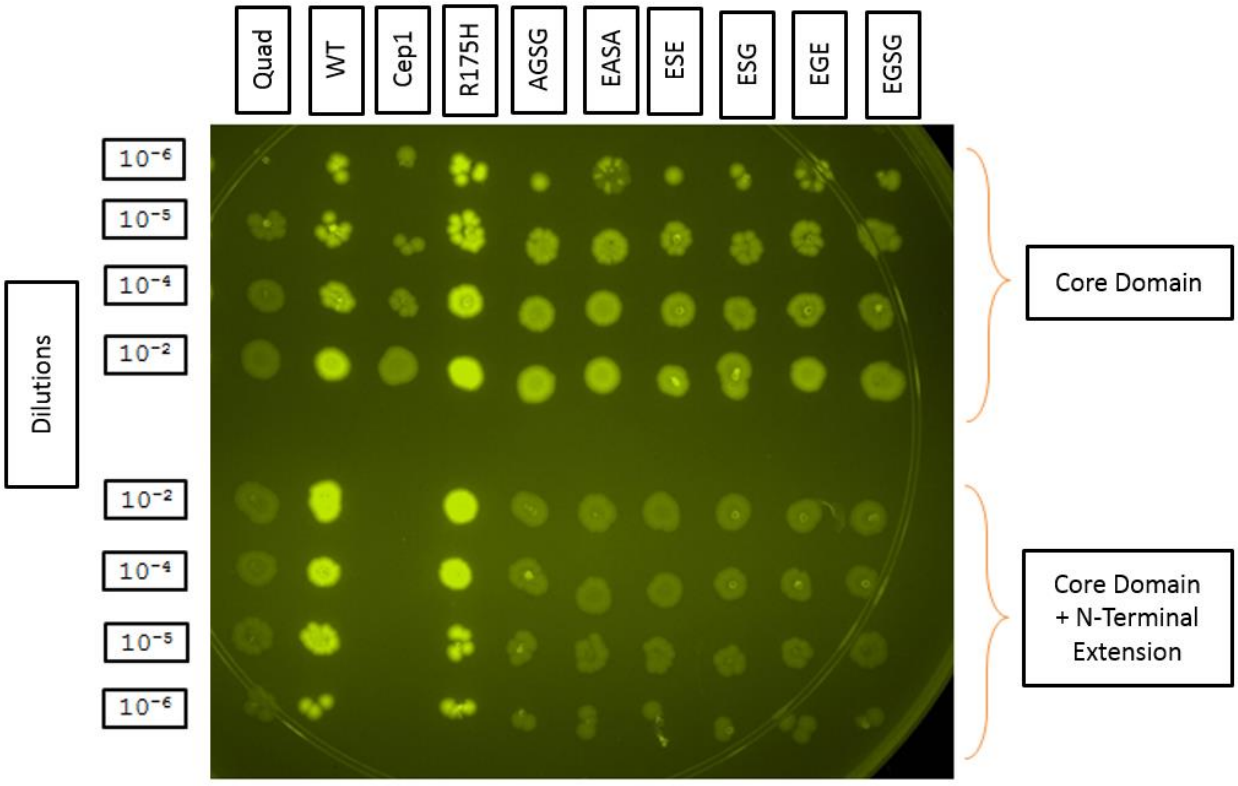

Figure 19: A picture under UV-light of a cell-based screen containing several variants of p53 with shortened S7S8 turns along with numerous controls. Saturated cultures were diluted from 10^{-2} to 10^{-6}.

The screen was done using double transformed DH10β cells with the p53 variant in pACBAD and GFP. The top half of the screen has all of the variants without the N-terminal extension and the bottom half has the variants with the N-terminal extension (Cep1 is the exception because it

does not have an N-terminal extension). Two variants clearly fail the screen: Wild Type p53 and R175H, both with and without the N-terminal extension. The fact that R175H fails is no surprise because mutation destabilizes Wild Type by more than 3 kcal/mol; however, it is surprising that Wild Type fails the screen, even with the extension. The Quad mutant passes the screen with and without the N-terminus and Cep1 passes too. The remainder of the variants – the six S7S8 turn mutants tested – all appear intermediate without the N-terminus and all pass the screen with the N-terminus.

Protein Expression and Purification

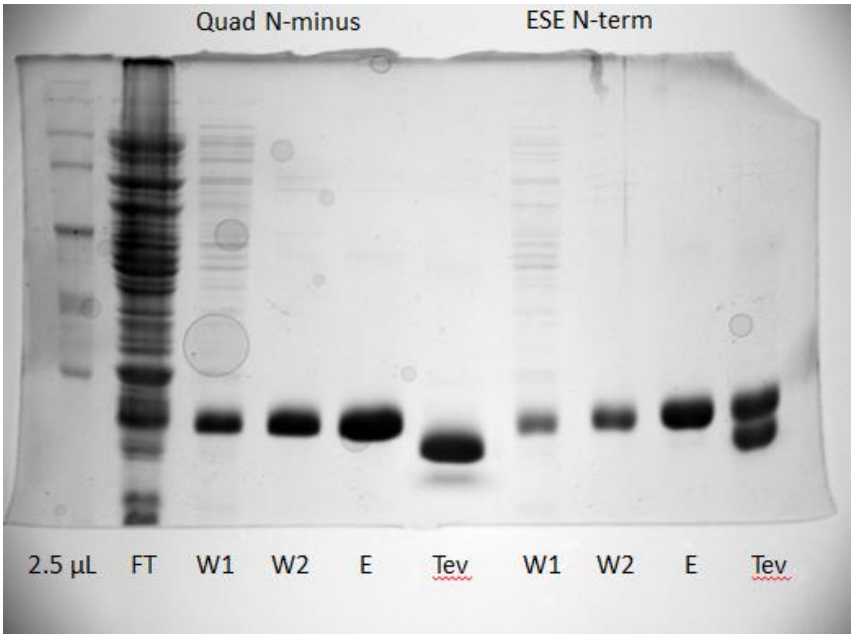

Figure 20: A 12.5% SDS-PAGE gel of two variants of p53 protein.

Biophysical Characterization

Biophysical characterization of N-minus Quad was performed by David Bowles.

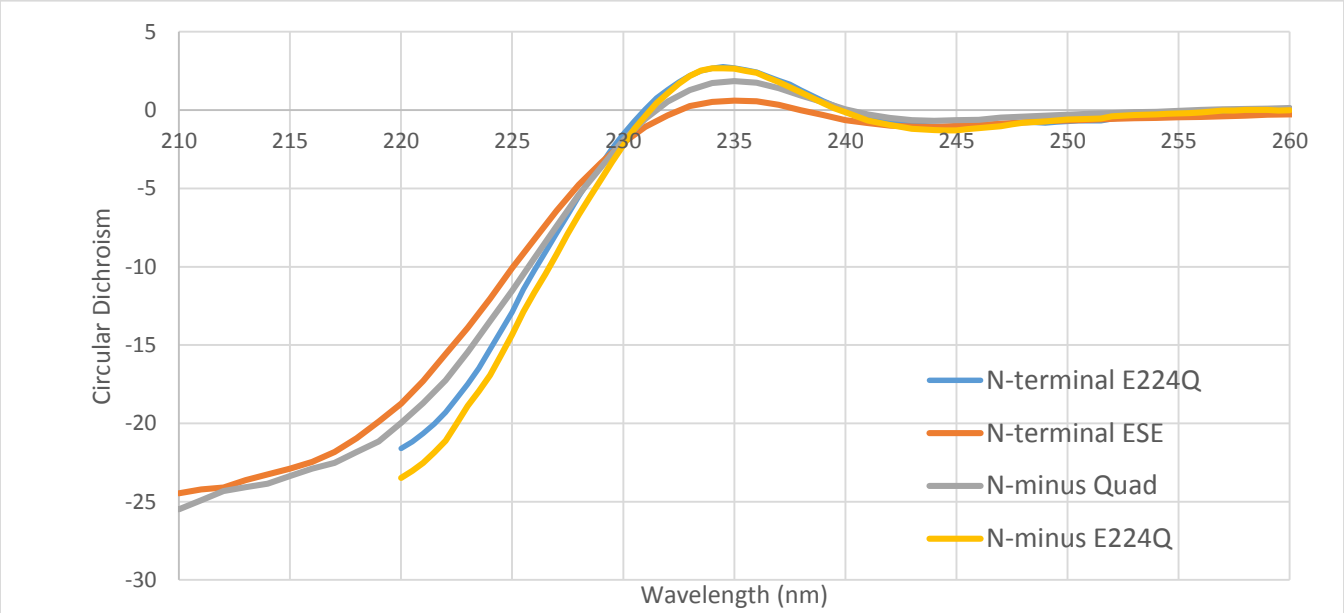

Figure 21: Circular Dichroism

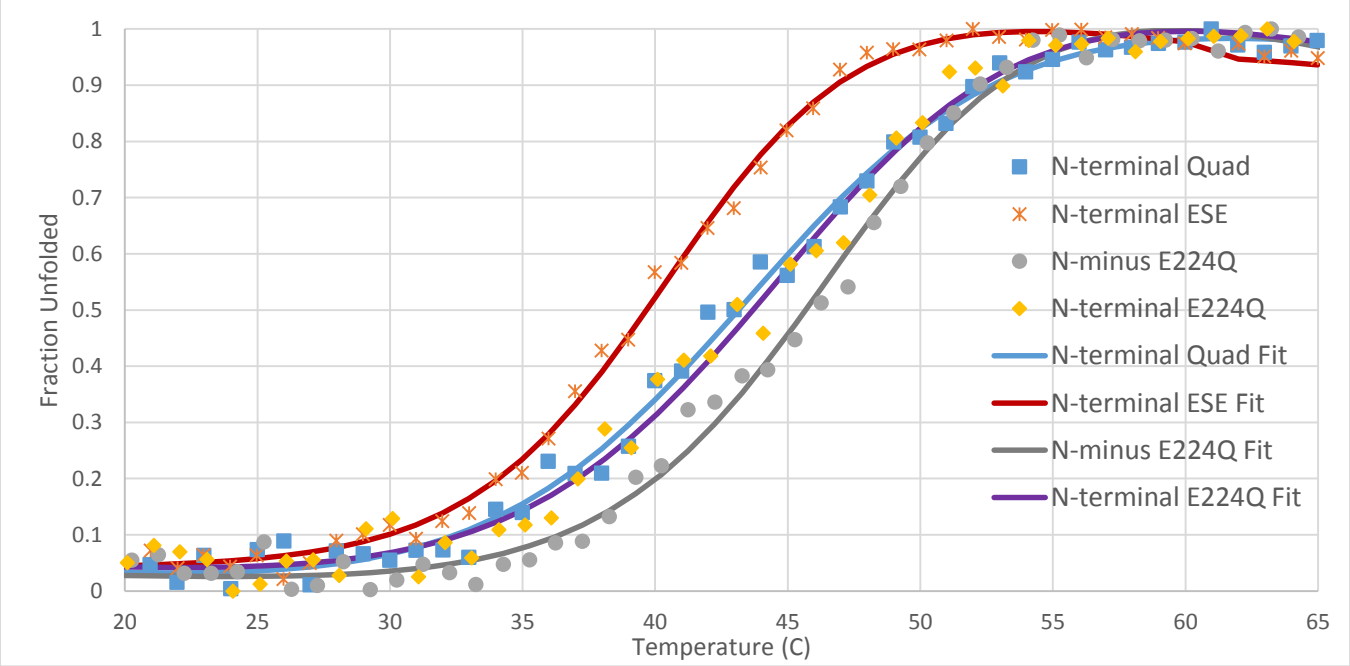

Figure 22: Thermal melt using circular dichroism at 222 nm

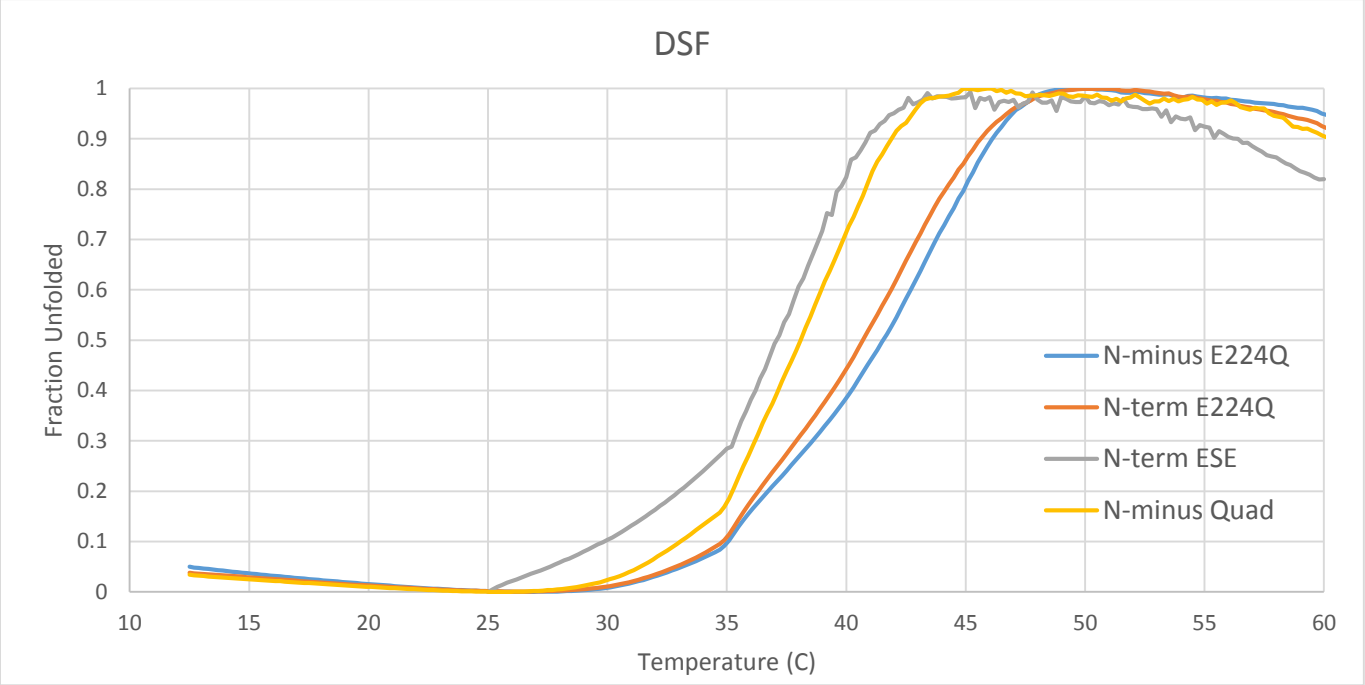

Figure 23: Thermal melt using Differential Scanning Fluorimetry

p53 Variant	N-minus CD Tm	N-terminal CD Tm	N-minus DSF Tm	N-terminal DSF Tm
WT	38.4	37.4	36.7	35.3
Quad	43.6	43.8	39.7	40.0
ESE	In Progress	39.6	In Progress	35.6
E224Q	45.3	41.6	47.2	40.3

Table 3: Table of Thermal Melt temperatures from CD and DSF

p53 Variant	S7S8 Sequence	N-minus Screen Activity	N-terminal Screen Activity
WT	YEPPEVGSDC	Inactive	Inactive
Cep1	MYPGA----V	Active	N/A
Quad	YEPPEVGSDC	Active	Active
R175H	YEPPEVGSDC	Inactive	Inactive
AGSG	YA----GSGC	Intermediate	Active
EASA	YE----ASAC	Intermediate	Active
EGE	YE-----GEC	Intermediate	Active
EGSD	YE----GSDC	Intermediate	Active
ESD	YE-----SDC	Intermediate	Active
ESE	YE-----SEC	Intermediate	Active
ESG	YE-----SGC	Intermediate	Active
EGSG	YE----GSGC	Intermediate	Active

Table 4: Summary of screen with GFP

*Cep1 does not have an S7S8 sequence; the sequence listed is its homologous region. R175H, like the S7S8 turn mutants, is made in the context of the Quad p53 mutant, which has the following mutations – M133L, V203A, N239Y, and N268D.

Western Blot

Figure 24: Western Blot with p53 purified protein

In order to evaluate if the Western Blot could be used, it was decided to use purified p53 protein for an initial Western Blot. Even though the ultimate goal of the Western is to detect variations of p53 protein in the screening vector and would have to be done with cell lysate, this Western Blot was used with purified protein to get a better sense if the Pab240 antibody would be applicable. These results, while giving a clearer image of the purified p53 protein (the bottom row on the membrane) were discouraging due to the high amount of background at the top of the membrane and the weak binding at lower concentrations. After this Western, the plan was to do one more Western Blot with much more primary antibody and more washes to improve binding and reduce background. If the results did not improve, it was thought that a flag tag could be used instead.

A

B

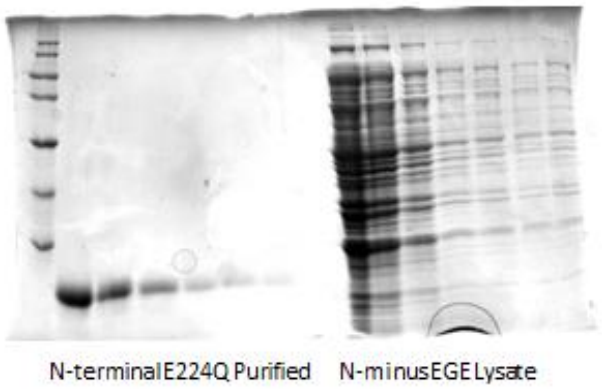

Figure 25A: Western Blot with purified N-terminal E224Q p53 protein on left and N-minus EGE p53 lysate on right

Figure 25B: Corresponding SDS-PAGE gel

While a 1:1000 dilution was used for the Pab240 primary antibody in the first two Western Blots, this Western had a 1:175 dilution, using 170 µg of antibody. Both purified p53 and lysate were used on this Western. A protein gel with equal amounts of protein is shown on the right. The left half of the protein gel was run with a serial dilution of purified N-terminal E224Q p53 and the right half was a serial dilution of N-minus EGE lysate. One complication faced in this Western was that the electrophoresis could only run at 150 V so it was run for 90 minutes instead of 80 minutes. Another problem was that the visualization was difficult to due malfunction in the Typhoon; this is why the picture is blurry. That being said, the Western worked well as a diagnostic tool. At first it was worrisome that there was a lot of white space for the purified p53, just as there was in the first Western; there was also more background above. However, when we realized that the white represented superfluous binding by the antibody, all three Westerns become much clearer. It then appeared that the background in the upper left of the membrane

could actually be oligomers – dimers, trimers, etc. of amyloid p53. It was also encouraging that the lysate was clearly visible on the Western, even at lower concentrations. Based upon this result, it was decided to run another Western with several variants of p53 lysate, including wild type, Quad, Cep1, ESE, and R175H (all with N-terminal and N-minus). The amount run was be based upon the lysate lanes in this Western. In addition, the Western was run with DAB, a browning agent, instead of chemiluminescence.

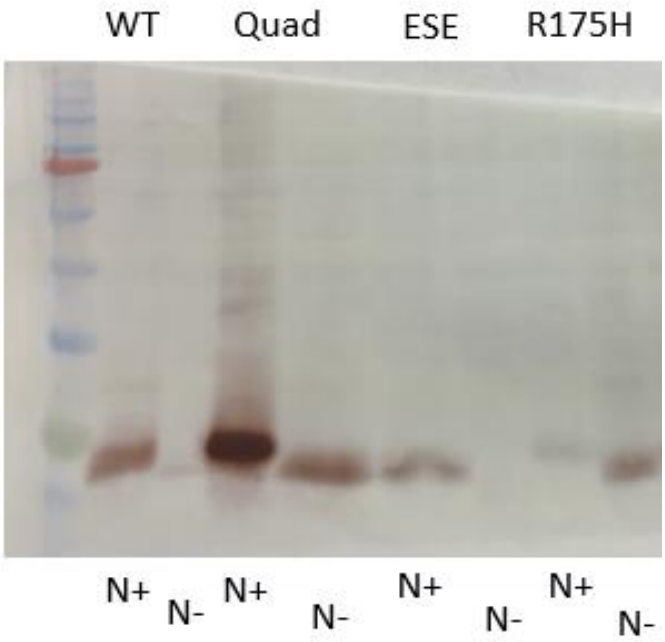

Figure 26: Western Blot with DAB

The Western was much easier to visualize with DAB than with chemiluminescence. It is advisable to stick with DAB for the future. This Western demonstrates that there is much more p53 protein present in the N-terminal variants of the screen (except for R175H), which helps explain why they pass the screen better in the S7S8 turn variants with the extension.

Conclusion

By the end of my research, three distinct questions had formed.

1. Can altering the S7S8 turn stabilize p53?
2. What is the effect of the N-terminal extension on the DNA-binding domain of p53?
3. Can we further characterize the screen?

S7S8 turn

The fact that we have created stable p53 mutants by shortening the S7S8 turn is encouraging. The mutants all appear intermediate on the screen without the N-terminal extension and then pass with the extension. CD spectrum and DSF of N-terminal ESE illustrate that it has similar unfolding curves to Quad and Wild Type p53. While I have created eight S7S8 turn mutants by shortening the turn by four or five residues, David Bowles has created a library of mutants in an effort to find the most stable mutants that pass our screen. Hopefully it will provide a number of more stable p53 variants.

N-terminal extension

Results on the effect of the N-terminal extension on p53 stability are a little unclear. On the one hand, the addition of the N-terminus raises the activity of all of the S7S8 turn mutants in the screen. On the other hand, the extension has not showed increase stability by any of the biophysical characterization methods. At this time, I would confidently say that the N-terminal extension does not increase the stability of the core domain.

Still, adding the N-terminal extension to our variants was very helpful. The main challenge in characterizing p53 variants is aggregation. When some of the proteins were being purified, they would aggregate immediately after adding lysis buffer. This was especially significant for the N-minus variants. One of the best techniques was to elute the protein into as much as 20 mL of p53 storage buffer because aggregation is dependent on concentration, temperature, and time. At first, Amicon tubes were centrifuged to concentrate the p53, but it had to be done at a low rpm and took a long time as a result. We switched to using Vivapores and aquicide (a much more cost-efficient method) for concentration. Both methods worked very well to quickly concentrate the p53 protein before analysis. In addition, the variants with the N-terminus went much longer without aggregating than the N-minus variants. While it may be unsatisfying that the N-terminal extension does not increase the core domain's stability, it can be a positive thing to know that the extension can be added to prevent aggregation without worry of affecting the dynamics of the protein.

Screen

There are a few questions surrounding the screen. Why doesn't Wild Type p53 pass (with or without the N-terminal extension)? Why does the addition of the N-terminal extension raise all of the S7S8 turn mutants from intermediates to clearly passing the screen? What precisely is the dynamic range of the screen?

These questions were attempted to be answered with Western Blots containing lysate from the same cultures that were plated on the screen. This was a challenge because p53, which we usually purify with a His-tag, does not have the tag in pACBAD so it cannot be purified. Therefore, the lysate must be run on the Western instead of purified protein.

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